

Attorney Docket No.: UMD-0070
Inventors: Langenfeld, John
Serial No.: 10/044,716
Filing Date: January 11, 2002
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Amendments to the Specification:

Please replace paragraph [0056] beginning at page 17, line 10, with the following rewritten paragraph:

--[0056] The present invention encompasses known antagonists of BMP-2 activity, including noggin (Brunet, L. J., et al., "Noggin, Cartilage Morphogenesis, and Joint Formation in the Mammalian Skeleton" Science 280(5368): 1455-7 (1998); U.S. Pat. No. 6,075,007, Economides, et al.), chordin (U.S. Pat. No. 5,896,056, LaVallie, et al.; Millet, C., et al., "The human chordin gene encodes several differentially spliced variants with distinct BMP opposing activities" Mech. Dev. 106(1-2): 85-96 (2001)), gremlin (GenBank GENBANK™ Accession No. AF154054), cerberus 1 homolog (GenBank GENBANK™ Accession No. NM_005454), and DAN.--

Please replace paragraph [0089] beginning at page 30, line 14, with the following rewritten paragraph:

--[0089] Applicant performed his initial experiments on normal and cancerous lung tissue and lung cancer cell lines. RT-PCR was performed using BMP-2 primers and showed expression in 9 out of 10 tumors examined. (Figure 2) ~~The RT-PCR products were separated electrophoretically on an ethidium-stained agarose gel and identified based on their migration rate. These separated RT-PCR produces were not sequenced, however, and the "BMP-2 primers" used were potentially capable of amplifying both BMP-2 and BMP-4, which are highly homologous. Thus, this data alone cannot be definitely interpreted as showing amplification of BMP-2 in the absence of sequencing data.~~ Using Western blot analysis,

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Applicant found that the mature active 14 kD BMP-2 protein was aberrantly expressed in almost all of the 25 non-small cell lung carcinoma (NSCLC) tissue specimens examined. There was little to no expression of BMP-2 in 11 normal lung tissue specimens. A representative Western blot is shown in FIG. 3. An anti-actin immunoblot showed near equal loading of the samples. (Figure 3(b)) In addition, BMP-2 was found to be highly expressed in all epithelial derived lung carcinomas of which NSCLC is derived and in the rare malignant neuroendocrine tumor. (Figure 3(c) and Figure 3(e), Lane 4, respectively) Western blot analysis of each of the different cell types comprising NSCLC-adeno, squamous, large cell, and bronchoalveolar carcinomas-revealed that the level of BMP-2 expression was not dependent on the cell type or whether the tumor was well or poorly differentiated. In comparison, the level of BMP-2 expression in benign lung tumors (Figure 3(e), Lane 1) and inflammatory diseases of the lung (Figure 7(a), Lane 1) was very low, similar to that seen in normal lung tissue, showing that BMP-2 is not an acute phase protein and that high levels of BMP-2 expression are indicative of malignant tumors. Neither BMP-4 nor BMP-7 expression was detected in the lung tissue specimens or the A549, H7249, IHBE, and NBE cell lines by Western analysis. (Figure 3(f))--

Please replace paragraph [00100] beginning at page 35, line 9, with the following rewritten paragraph:

--[00100] In addition, Applicants showed that BMP-2 stimulates the migration and invasion of the human lung cancer cell lines A549 and H7249. In one assay, recombinant BMP-2 caused a dose responsive increase in migration of cells from ~~transwell~~

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TRANSWELL™ migration chambers. (Figure 13(a)) In another, BMP-2 stimulated the migration of A549 and H7249 cells cultured on glass cover slips toward Affi-blue agarose beads containing recombinant BMP-2. (Figure 13(c) and (d)) In addition, using ~~transwell~~ TRANSWELL™ chambers coated with ~~Matrigel~~ MATRIGEL™, Applicants also showed that recombinant BMP-2 caused a dose responsive increase in the invasion of both A549 and H7249 cells. (Figure 13(e))--

Please replace paragraph [00104] beginning at page 36, line 14, with the following rewritten paragraph:

--[00104] Applicants also found that noggin completely inhibited the ability of BMP-2, discussed above, to enhance the migration of the A549 cells in a ~~transwell~~ TRANSWELL™ chamber. (Figure 13(b))--

Please replace paragraphs [00105] and [00106] beginning at page 36, line 20, with the following rewritten paragraphs:

--[00105] Representational difference analysis (RDA) subtraction technique was used to identify genes highly expressed in a non-small cell lung carcinoma obtained from a patient (tester) in comparison to normal bronchial human epithelial cells (driver). The technique for RDA described in the following references was followed: Holmes, M. L., et al., Molecular and Cellular Biology 19: 4182-4190 (1999); Hubank, M., Nucleic Acids Research 22:5640-5648 (1994). Normal human bronchial epithelial cells were purchased from ~~Clonetics~~ CLONETICS™, BioWhitaker (Walkersville, Maryland) and were maintained in serum free media. Human tissue

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specimens were obtained directly from the operating room and immediately frozen in liquid nitrogen. Tissue was stored in liquid nitrogen at -70°C.

[00106] To perform RDA, mRNA was purified from the samples using Oligo dT columns (~~Pharmacia~~ PHARMACIA™, Peapack, NJ) according to the manufacturer's instructions and cDNA was then obtained using the ~~Pharmacia-Time Saver~~ PHARMACIA™ TIMESAVER™ cDNA synthesis kit also according to the manufacturer's instructions. cDNA was digested with Sau3A I endonuclease, R-linker ligated, and amplified by PCR. The R-linkers were removed and J-linkers ligated to the tester. The driver and tester cDNA were hybridized at 67 C for 20 hours (driver in excess 100:1) and the subtracted tester cDNA amplified by PCR. A second round of subtraction was performed using N-linkers (driver in excess 800,000:1). The amplified PCR products were cloned into blue script and sequenced using a ~~ABI-Prism~~ an ABI PRISM® 377 DNA sequencer. Known genes corresponding to the subtracted tumor cDNA were identified by a BLAST database search.--

Please replace paragraphs [00108] and [00109] beginning at page 38, line 3, with the following rewritten paragraphs:

--[00108] Applicant detected expression of BMP and BMP receptors in a number of normal and cancerous tissue specimens and cells. As described above, all human tissue specimens were obtained directly from the operating room and immediately frozen in liquid nitrogen and stored at -70 C. Normal human bronchial epithelial (NBE) cells were purchased from ~~Clonetics~~ CLONETICS™, BioWhitaker (Walkersville, Maryland) and were maintained in serum free media. Immortalized human bronchial epithelial (IHBE), BEAS-2B, cells

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were derived from normal bronchial epithelial cells immortalized with an adenovirus-12-5V40 hybrid virus (32). A549 and H7249 are highly invasive human lung cancer cell lines. The cell lines were cultured in 5% fetal bovine serum (FBS) in Dulbecco's Modified Eagles medium (DME) containing penicillin, streptomycin, and glutamine with 5% ~~pCO₂~~ pCO₂ at 37°C. Western blot analysis was used to detect expression of the BMP ligand and its receptors in all of these samples. Immunohistochemistry studies were performed to detect BMP in non-small cell lung carcinoma samples and normal lung tissue samples derived from patients.

Western Blot Analysis

[00109] In preparation for Western blot analysis, cells were lysed in a modified RIPA buffer containing 150 ml NaCl, 50 ml tris, pH 7.5, 1% NP 40, 10% deoxycholic acid, and protease inhibitor cocktail from ~~Calbiochem~~ CALBIOCHEM™. Tissue specimens were sonicated on ice in the same modified RIPA buffer. The protein concentration of the resulting samples was measured using the Bradford assay technique. Recombinant human BMP-2, purchased from R & D Systems and reconstituted in PBS with gelatin, served as a control. Total cellular protein of the samples and recombinant human BMP-2 were analyzed by SDS-PAGE, transferred to nitrocellulose filter (~~Schleicher and Schuell~~ SCHLEICHER AND SCHUELL™, Keene, NH) at 35 V for 16 hours at ~~40°C~~ 4°C and then incubated with the desired primary antibody. Specific proteins were detected using the enhanced chemiluminescence system (~~Amersham~~ AMERSHAM™, Arlington Heights, IL).--

Please replace paragraphs [0104]-[0108] beginning at page 41, line 15, with the following rewritten paragraphs:

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--[0104] The sandwich ELISA method was used to determine VEGF concentrations in the cell culture media of A549 cells treated with noggin and in the cell culture media of human aortic endothelial cells treated with BMP-2. 100 ~~ul~~ uL of the monoclonal capture antibody, diluted in carbonate buffer (sodium bicarbonate, sodium carbonate, pH 9.0), was added to each well of a MaxiSorb Nunc-Immuno plate and incubated overnight at 4 C. The plates were washed two times with washing buffer (1x PBS with 0.0005% ~~tween-20~~ TWEENTM-20). Then, 200 ~~ul~~ uL of blocking buffer (1x PBS with 1% BSA) was added per well and incubated for 2 hours at room temperature. The plates were then washed 4 times with washing buffer.

[0105] The recombinant protein standards and samples (100~~ul~~ uL per well) were added and the plate was then incubated overnight at 4 C. The plates were washed 5 times with washing buffer. The biotinylated detection antibody was diluted in incubation buffer (1x PBS with 10% fetal bovine serum) for a final concentration of 1 ~~ug~~ ug/ml. 100 ~~ul~~ uL of the detection antibody was added per well and incubated for 1 hour on a shaker at room temperature. The plates were washed 6 times with washing buffer and 100 ~~ul~~ uL of streptavidin-HRP conjugate (1:10,000) was added per well. The plates were incubated for 45 minutes at room temperature on a shaker and then washed 6 times with washing buffer. 100 ~~ul~~ uL/well of the substrate reagent (0.2 M citrate buffer, 1 mg/ml o-phenylenediamine dihydrochloride (OPG), 3% hydrogen peroxide) was added and covered with aluminum foil for ten minutes. The reaction was stopped with 100 ~~ul~~ uL/well of 2M sulfuric acid and absorbance determined using an automated plate

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reader with a 490/690 filter. The protein concentration was then determined from the standard curve.

**Example 7: Identification of BMP-2 as a Stimulant of Human Lung
Cancer Cell Migration and Invasion**

Migration Assay in Monolayer Culture

[0106] To detect BMP-induced migration in a monolayer culture, recombinant human BMP-2 (R & D systems, Minneapolis, MN) was coated to Affi-Blue agarose beads (~~Bio-Rad~~ BIO-RAD®, Hercules, CA) as described in the literature. (Vainio, S., et al., Cell 75: 45-58 (1993); Sloan, A. J., et al., Arch Oral Biol. 44: 149-156 (1999)) Briefly, 100 ml of the Affi-Blue agarose beads were incubated with either 10 ml of recombinant BMP2 reconstituted in PBS with gelatin (100 mg/ml) or PBS alone at 37°C for 2 hours, washed with PBS, and reconstituted with 40 ml of PBS. Glass cover slips were coated with serum free media containing BSA, fibronectin and collagen (32) and 50,000 cells were plated per cover slip in serum free media. Two microliters of the Affi-Blue agarose beads coated with recombinant BMP-2 or dilution buffer were placed in linear fashion next to the cover slips.

Chemotactic Assay

[0107] In the chemotactic assay, fifty thousand cells were placed in the upper chamber of an 8 micron ~~transwell~~ TRANSWELL™ migration chamber (Becton Dickinson, Bedford, MA) and 300 ml of serum free media with 0 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml recombinant human BMP-2 placed in the lower well. After 24 hours the filters were then removed and the top of the filter wiped with a cotton swab and the cells that migrated through the filters were stained with Syto-16 intercalating dye. Five high power fields were counted using fluorescent microscopy.

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To show that noggin inhibits BMP-2 induced migration, the experiment was also performed with each of the following in the lower well of the ~~transwell~~ TRANSWELL™ chamber: media alone, recombinant BMP-2 (500 ng/ml), and noggin (10 ~~ug~~ µg/ml) with recombinant BMP-2 (500 ng/ml).

Matrigel MATRIGEL™ Invasion Assay

[0108] Invasion was studied using ~~transwell~~ TRANSWELL™ chambers coated with 100 µl of ~~Matrigel~~ MATRIGEL™ (Becton Dickinson). Fifty thousand cells were placed in the upper chamber and 300 µl of serum free media with 0 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml recombinant BMP-2 placed in the lower wells. After 48 hours the ~~Matrigel~~ MATRIGEL™ was removed and cells that had migrated through the filter were stained with Syto-16 intercalating dye and 5 high power fields counted using fluorescent microscopy.--